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Acute toxicity bioassay in sodium fluoride exposed Zebrafish (*Danio rerio*)

Mohan Kumar[1], Kundan Patel[2*] & G. B. Chand[3]

[1-3]*Aquatic Toxicological Laboratory, Department of Zoology, Patna University, Patna-800005, Bihar, India.*
Corresponding Author (Kundan Patel) Email: kundanpatel125@gmail.com[*]

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ABSTRACT

Background: Fluoride finds its entry into the aquatic organism through skin and gills through adsorption, apart from inducing a plethora of deleterious impact. It also enters the food chain and creates wreak havoc in the organisms of the higher trophic level. The studies related to acute toxicity bioassay of sodium fluoride to different freshwater fish species and related histopathological anomalies are still in its infancy stage and needs to be properly addressed.

Methods: In the present study, Zebrafish, *Danio rerio*, were treated with different concentrations of sodium fluoride for 24, 48, 72 and 96 hours. The physico-chemical parameters of the water were measured as per standard protocol of APHA. The Median lethal concentration of sodium fluoride was determined by probit analysis and it was confirmed using pilot test. Any change in pattern of locomotion and behaviour observed was properly monitored.

Results: A dose- and duration-dependent increase in restlessness and rapid abnormal movement were common in fish during treatment. The LC_{50} for 48 hours and 96 hours were determined as 295.12 mg/L and 257.039 mg/L, respectively.

Conclusions: The bioassay of LC_{50} of any xenobiotic to the experimental fish is basic requirement to study the acute, sub-acute and chronic toxicity impact of the xenobiotic. The findings of this paper envisage the scientific LC_{50} value of sodium fluoride to *Danio rerio* and it will help in deciding the dose and duration for the toxicity assessment of sodium fluoride to the fish.

Keywords: APHA; Bioassay; *Danio rerio*; Freshwater fish; LC_{50}; Median lethal concentration; Pilot test; Probit analysis; Toxicity.

1. Introduction

Aquatic fluoride toxicity has emerged as a global issue due to its hazardous impact on the aquatic organism as well as human beings [1]. Fish is considered as the cheapest and best proteinaceous supplement of protein in the developing countries. The fluoride enters in the piscivorine community through food chain [1-3]. Fluoride enters the body of the fish through gills and skin and tends to be deposited in skeletal elements, skin, gills, liver, gut and muscles [3,4]. Due to its high electronegativity and solubility, fluorine is usually found in the form of fluoride, which may be organic or inorganic [5,6]. Fluoride is one of those compounds which is required by the body up to a limit, but at higher level it leads to various anomalies [7-10]. Some of the natural sources of fluoride include the weathering of minerals containing fluoride, that remain present in the earth's crust like fluorapatite, fluorite, cryolite, *etc.* as well as volcanic eruptions releasing gases like hydrogen fluoride [7,11]. It is also present in large amount in thermal waters having high pH [12]. It mainly enters the body through drinking water [8]. In India, about 14 Indian states, namely - Andhra Pradesh, W.B., Bihar, Odisha, Rajasthan, M. P., Punjab, Gujarat, Haryana, T. N., Karnataka, Uttar Pradesh, Kerala and Maharashtra are facing the problem of high content of fluoride in water [13,14].

Fluoride-contaminated water is being consumed by more than 200 million people in different regions of the world [13]. People of many countries are suffering from fluorosis [6, 15].

Sodium fluoride has been shown to reduce the protein content in several organs like muscles of gills, brain and kidneys. However, an increase in the protein content in liver of *Labeo rohita* have also been reported [7]. The toxic

effects of elevated levels of fluoride on physiological and biochemical parameters of different fish species have been well documented by Sigler and Neuhold (1972), Pillai and Mane (1984), Gikunju (1992) and Azmat *et al.*, 2012 [16-19].

Toxicity assessment of a particular xenobiotic on aquatic organism predicts the health status of the water body and can be considered as major screening tool for predicting strategies for protection and conservation of an aquatic system. It also helps us to determine the maximum permissible limit of the toxicants in an ecosystem. Zebrafish is widely used for such studies in aquatic ecosystem [6]. Acute toxicity test is one of the most pertinent toxicological tests to determine the concentration of the test material on a group of a test organism during short term exposure under controlled condition [20]. Among several techniques available for short term toxicity assessment, the determination of median tolerance limit (TLM) or median lethal concentration (LC_{50}) by probit analysis (statistical bioassay) is so far the most widely accepted techniques [21-26]. In the present study, acute toxicity assessment and related behavioral responses induced by sodium fluoride in freshwater Zebrafish, *Danio rerio* have been studied.

1.1. Study Objectives

(i) Assessment of acute toxicity of sodium fluoride to freshwater fish *Danio rerio.*

(ii) Determination of LC_{50} of sodium fluoride by graphical interpolation method.

(iii) Statistical bioassay of 24, 48, 72 and 96 hrs. LC_{50} of sodium fluoride by probit analysis.

(iv) Interpreting behavioural responses in zebrafish induced by sodium fluoride.

(v) Correlating physico-chemical condition of water and acute toxicity of sodium fluoride to the fish.

2. Materials and Methods

2.1. Procurement of fish

Freshwater fish, *Danio rerio*, weighing 410±50 mg and length 3.4±0.5cm were procured from Howrah Fish market in October, 2021 through local supplier.

2.2. Acclimatization in the laboratory

They were properly transported to the laboratory, given prophylactic treatment of 0.1% $KMnO_4$ and then kept in aquaria of 90 L capacity. Each of the aquaria contained adequately aerated and dechlorinated water. They were acclimatized in the laboratory for 15 days. During acclimatization, water was changed after every 24 hours and were fed *ad libitum* @ 2-3% of body weight.

2.3. Experimental Design

In the experimental protocol, sodium fluoride of Merck Pvt. Ltd. was used. Test solution was prepared using deionized water. Preparation of stock solution was done following the standard procedure of laboratory manual.

2.4. Acute toxicity test

It was performed following the standard method of APHA (2005). During the test period, the fish were kept on starvation.

2.5. Range-finding test

The test was conducted in aquaria to determine the concentration of sodium fluoride in the short-term test for finding out LC_{50} range. The test fish were exposed to different concentrations of sodium fluoride starting from 0 to 600 mg/L with an interval of 50 mg/L till the observation of 100 % mortality.

2.6. Median Lethal Concentration (LC_{50}) Test

Based on the result of range-finding test, a definitive acute toxicity bioassay was conducted by exposing the fish to different concentrations of sodium fluoride along with one control to get the LC_{50} concentration. A stock solution was prepared by dissolving 1.00 g of sodium fluoride in one litre of distilled water. Test concentrations were prepared by diluting appropriate aliquots of the stock solution in groundwater. Each concentration was set in triplicates with 10 fish (average weight 410±50 mg and average length 3.4±0.5 cm) per aquarium. Feeding was stopped 24 hours prior to the start of 96 hours LC_{50} experiment and fish were starved during the entire study period. The whole experiment was conducted under the natural 12-hr photo period. At the end of every 24 hours, water was changed in aquaria and adequate amount of sodium fluoride stock solution was added to maintain the exact concentration of the aquarium. Sodium fluoride being white powder that is readily soluble in water and hence its addition in the aquarium made the water turbid. The fish were monitored carefully to observe any specific changes in behavior and other responses like swimming pattern, surfacing activity, opercula movements. They were considered to be dead when there was no any visible movement (*e.g*., no opercular motion), and on touching the caudal peduncle produced no reaction. The mortality count was properly maintained after every 24, 48, 72 and 96 hours. The dead fish were removed from aquaria immediately without delay. The surviving fish were counted after the scheduled duration of each exposure.

2.7. Statistical analysis

LC_{50} value was calculated using Probit regression methods [25]. For this calculation, logarithm of concentrations was used to obtain regression lines. We used standard protocol of UNEP/FAO/IAEA (1987) to calculate P-value, upper and lower confidential limit of the regression line and also calculated chi-square value.

3. Results and Discussions

3.1. Behavioral changes in exposed fish

Sodium fluoride exposed *Danio rerio* showed abnormal behavioral patterns such as swimming near the upper surface of water, gathering near aerator, and frequent surface-to-bottom movements, faster opercula activity, surfacing and gulping of air. The exposed fish were more active and showed erratic swimming in comparison to control fish, which were calm and settle at the bottom of aquaria during test. Prior to death, the exposed fish became inactive, turned upside down and settled down to the bottom of aquarium. Fish with higher dose showed dark pigmentation in their ventral region. The intensity of change in behavioral pattern increased with increase in concentration of sodium fluoride in water. The changes in the behaviour might be due to the hyper activity of the neural mechanism or secondary stress response in the fish due to the release of stress hormones from respective endocrine glands in response to sodium fluoride-induced stress. The behavioral changes observed in the present

study are in agreement with the findings of previous conducted studies [27]. Physico-chemical parameters of water of aquaria were tested and are shown in Table 1. The checklist of test condition and procedure conducted for 96 hours LC_{50} studies have been shown in Table 2.

Table 1. Physico-chemical parameters of test water

1.	pH	7.57
2.	TDS	1029 mg/l
3.	Total Hardness	310.86 ppm
4.	Alkalinity	455.4 mg/l
5.	Fluoride	0.57 ppm
6.	Water Temperature	28±2 ºC
7.	Room Temperature	28±3 ºC

Table 2. Checklist of test conditions and procedures conducted for 96 hours LC_{50} study

Test Type	Static, 96-hr duration
Control water	Ground water with 0.05 mg/L fluoride level
Fish	*Danio rerio* with average weight 410±0.50 mg and average length 3.5±0.5 cm, 10 fish in each aquarium
Aeration	24 hours supply of aeration using aerators
pH	Suitable pH condition with near neutral pH value
Feeding	Feeding stopped 24 hours before the exposure of sodium fluoride
Observations	Mortality count and behavioral changes, twice a day up to 96 hours post-treatment
Measurements	Daily observation of water temperature, pH, DO
Endpoints	As specified in the test objective; 96-hrs LC_{50} (95% confidence limits)
Test validity	Invalid if >10% of control fish die or exhibit a typical/stressed behavior then % mortality corrected using Schneider-Orelli's formula

3.2. Mortality count

Concentration dependent mortality of fish has been observed. A trend of increasing mortality with increasing concentration of sodium fluoride has been obtained (Table 3). Logarithm of concentration of sodium fluoride and logarithm of fish mortality (Probit) show linear relationship in case of all the groups of treated fish (Table 4).

Table 3. Mortality count in fish at different concentrations of sodium fluoride

S. No.	Sodium fluoride concentration mg/ml	Mortality count after 24 hours (%)	Mortality count after 48 hours (%)	Mortality count after 72 hours (%)	Mortality count after 96 hours (%)
1.	250	20	20	20	40
2.	300	20	50	60	60
3.	350	50	90	90	90
4.	400	60	90	100	100
5.	500	90	100	100	100
6.	600	100	100	100	100

Table 4. Assessment of acute toxicity showing tolerance of *Danio rerio* to sodium fluoride

Exposure period (hours)	LC_{20} value (in ppm)	LC_{50} value (in ppm)	LC_{90} value (in ppm)	Regression equation Y=ax+c	95% confidence limit		P-value	Chi-square value	
					Upper limit	Lower limit		Calculated value	Table value
24 hours	288.403	338.844	436.515	11.82X-24.89	-6.82	-42.97	0.0187	4.2852	7.815
48 hours	257.040	295.121	363.078	13.74X-28.90	-16.94	-40.85	0.0026	2.8952	7.815
72 hours	234.423	269.153	331.131	13.99X-29.04	-7.18	-50.91	0.0210	2.7355	7.815
96 hours	223.872	257.039	323.593	12.88X-26.09	-4.94	-47.25	0.0267	1.3983	7.815

The 24 hours, 48 hours, 72 hours and 96 hours LC_{50} of *Danio rerio* for sodium fluoride were calculated as 338.84 ppm, 295.12 ppm, 269.15 ppm and 257.04 ppm, respectively using Probit regression method. From the above regression equation mentioned in Table 4, it is highly evident that mortality increased with the increase of exposure period (Figures 1-4). The median lethal concentration has been found to vary from species to species and the initial hardness of water also affects it [7].

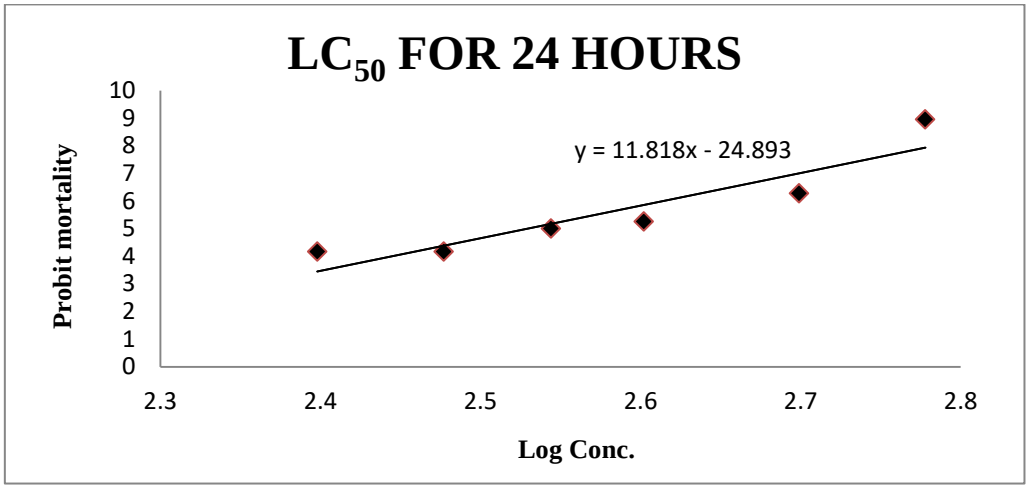

Figure 1. Regression line based on probit analysis of $\log_{10}$ of concentration of sodium fluoride vs. probit of percent mortality of *D. rerio* at 24 hours

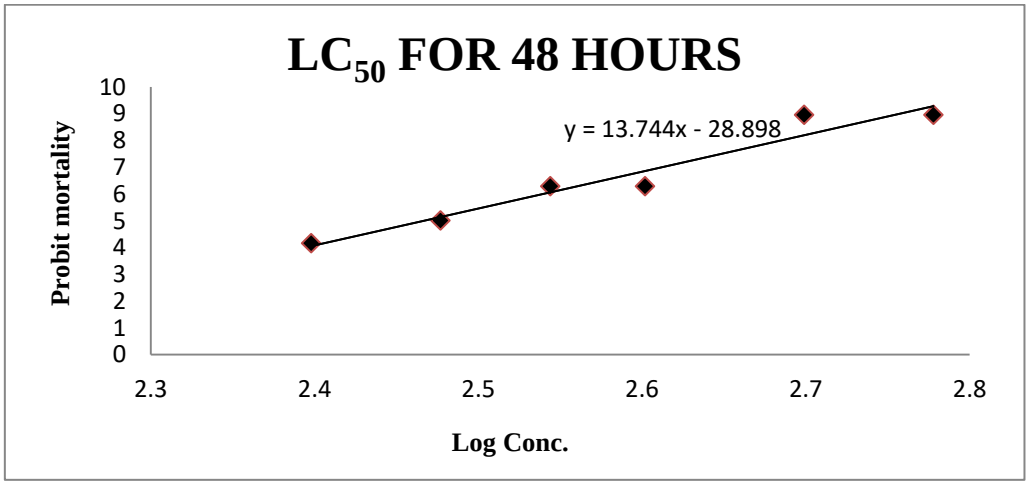

Figure 2. Regression line based on probit analysis of $\log_{10}$ of concentration of sodium fluoride vs. probit of percent mortality of *D. rerio* at 48 hours

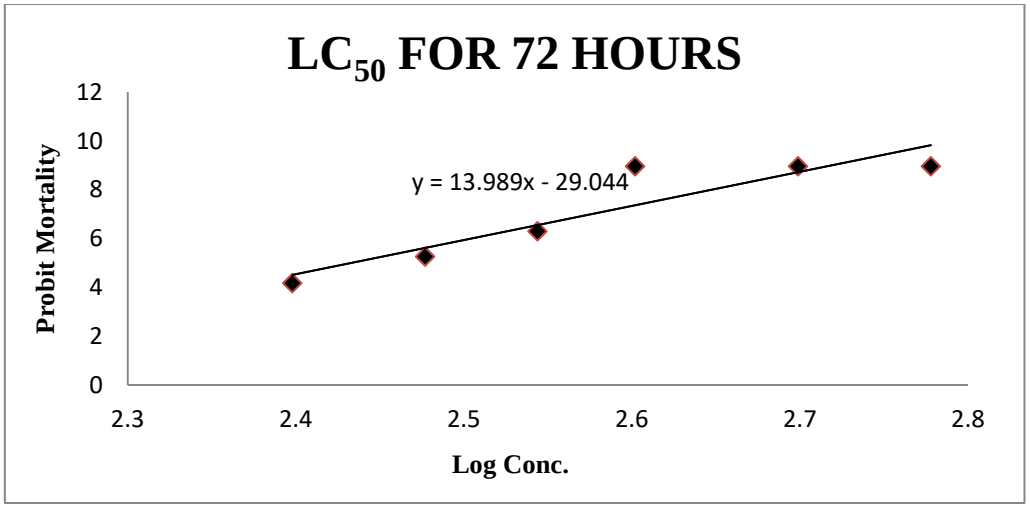

Figure 3. Regression line based on probit analysis of $\log_{10}$ of concentration of sodium fluoride vs. probit of percent mortality of *D. rerio* at 72 hours

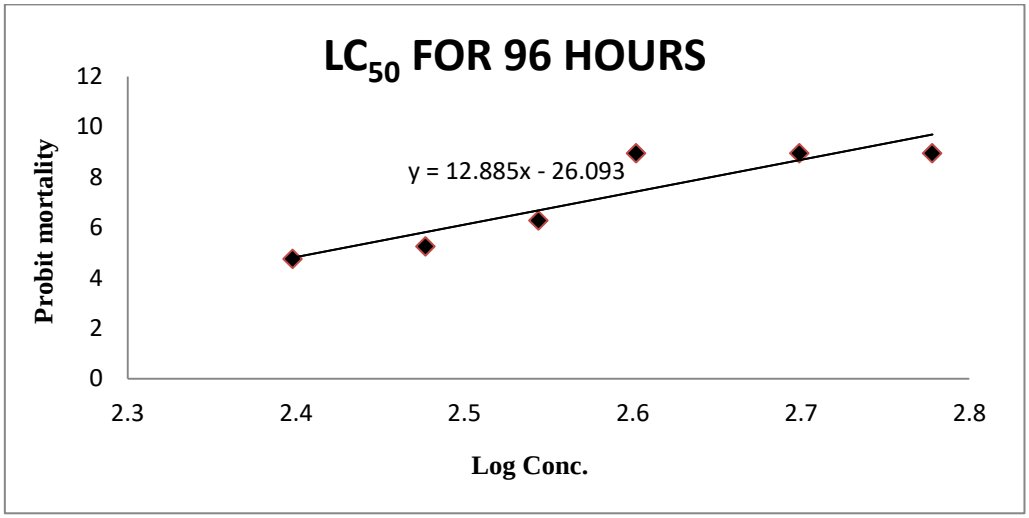

Figure 4. Regression line based on probit analysis of $\log_{10}$ of concentration of sodium fluoride vs. probit of percent mortality of *D. rerio* at 96 hours

It has been observed that the calculated value of LC_{50} for the same toxicant may differ from species to species due to their mode of action [28]. Witeska and Jeezierska (2003) found that water parameters such as dissolved oxygen concentration, temperature, total hardness, total alkalinity, fish species influence the toxicity levels for fish [29]. According to the earlier studies, highly variable 96-hr LC_{50} values have been reported for fluoride in different species of fish. In *Cyprinus carpio*, at water temperature 22.0-24.0 ºC and hardness 20.0 mg/L, the 96-hr LC_{50} was 321.6 mg/L [30].

In case of *Heteropneustes fossilis* (Bloch), at temperature 27±1.5 ºC and hardness 250-290 mg/L, the 96-hr LC_{50} was found to be 350 mg/L [31]. In tilapia (*Oreochromis mossambicus*), the 96-hr LC_{50} of Sodium fluoride was calculated as 54.0 mg/L [32]. The 96-hr LC_{50} value for brown trout at temperature 16.1±0.13 ºC was observed as 160.5 mg/L [33]. The 96-hr LC_{50} value of sodium fluoride in *Puntius sophore* was found to be 126.12 mg/L at water temperature of 18.33±0.07 ºC and hardness of 291.25±1.89 mg/L [27].

With the variation in water temperature and the hardness, it has been reported that the LC_{50} value changes even among the same species. In the rainbow trout fish the value of 96-hr LC_{50} was found to be 107.5 mg/L at a water temperature of 15.3±0.22 ºC for sodium fluoride [33]. The 96-hr LC_{50} for the same fish exposed to fluoride in water with temperature 8 ºC and hardness 17 mg/L was found as 51 mg/L whereas at water hardness level 49, 182 and 385 mg/L, the fluoride tolerance limit was found to be 128, 140 and 193 mg/L respectively [34]. In *Labeo rohita*, the value of 96-hr LC_{50} was recorded as 935 mg/L at water temperature of ranging from 18-30 ºC [35], whereas at water temperature of 28±2 ºC and hardness 232 mg/L, it was found as 334.88 mg/L [7].

At the molecular level, it has been reported that sodium fluoride induces an increase in the release of cytochrome c (cyt C) from the mitochondria to the cytosol [10,36]. Exposure to sodium fluoride results in decreasing the ATP production, which further increases the cellular levels of ADP, AMP and GDP. Persistent inhibition of ATP production results in endogenous induction and activation of nitric oxide (NO). Nitric oxide inhibits mitochondrial respiration by decreasing the apparent affinity of cyt C for oxygen. Nitric oxide induction and activation has been well documented in cases of fluoride exposure [37-39]. Nitric oxide acts as oxygen ion quencher. Neither NO nor

oxygen ion is a strong oxidant, but NO and oxygen ion react to produce peroxynitrite, which is a strong versatile oxidant that can attack a wide range of biological targets and inactivates the enzyme action [40].

4. Conclusion & Future Recommendations

The findings of the present study reveal the statistical bioassay of LC_{50} of sodium fluoride to the freshwater Zebrafish (*Danio rerio*) based upon the most authenticated probit analysis method. It also highlights the sodium fluoride-induced behavioural and stress responses in Zebrafish. Assessment of acute toxicity of sodium fluoride on Zebrafish can be interpolated to human beings. The environmental relevance of the xenobiotic can be correlated with the LC_{50} of the toxicant in determining doses for experimental studies. The results of the present study furnish basic information for the further study of various toxicological hazards of sodium fluoride on the fish at molecular level. In the future, environmental concentration of sodium fluoride in the ground as well as surface water of different aquatic resources needs to be closely monitored. It is recommended to study the interaction of various external factors on the deleterious impact of sodium fluoride on organisms. An intensive scientific study of impact of fluoride on various biological aspects at molecular level of aquatic animals is needed. Bioaccumulation of fluoride at different trophic levels of aquatic food chain should be studied. Studies focused on finding antidotes and treatment for affected population needs the collaboration of various stakeholders including people engaged in health sector and scientists.

Declarations

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Competing Interests Statement

The authors have declared that no competing financial, professional or personal interests exist.

Consent for publication

All authors contributed to the manuscript and consented to the publication of this research work.

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